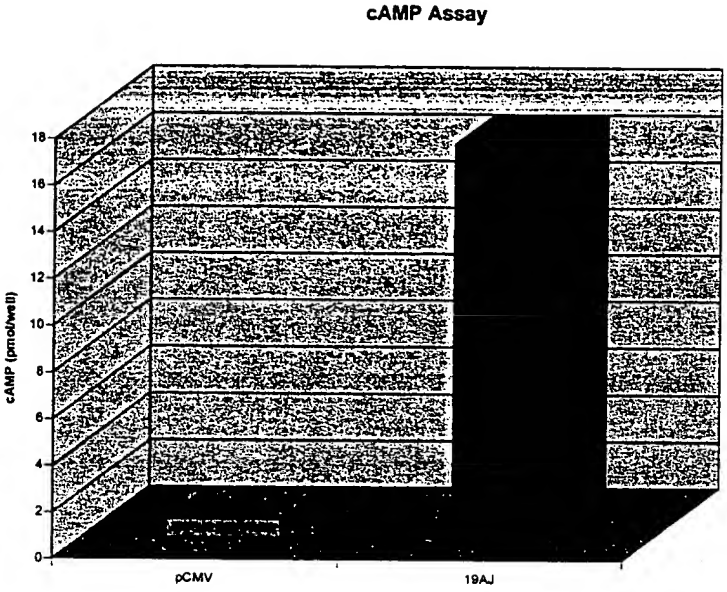
Appendix B
(B1-B8)

Appendix B1

cAMP Assay Protocol

In the following assay, a 96-well Adenylyl Cyclase Activation Flashplate was used (NEN: #SMP004A). First, 50ul of the standards for the assay were added to the plate, in duplicate, ranging from concentrations of 50pmol to zero pmol cAMP per well. The standard cAMP (NEN: #SMP004A) was reconstituted in water, and serial dilutions were made using 1xPBS (Irvine Scientific: #9240). Next, 50ul of the stimulation buffer (NEN: #SMP004A) was added to all wells. In the case of using compounds to measure activation or inactivation of cAMP, 10ul of each compound, diluted in water, was added to its respective well, in triplicate. Various final concentrations used range from 1uM up to 1mM. 10uM of dopamine was used in the assay. Next, the 293 cells transfected with 12ug (per 150mm tissue culture plate) of the respective cDNA (CMV or 19AJ) were harvested 24 hours post-transfection. The media was aspirated and the cells washed once with 1xPBS. Then 5ml of 1xPBS was added to the cells along with 3ml of cell dissociation buffer (Sigma: #C-1544). The detached cells were transferred to a centrifuge tube and centrifuged at room temperature for five minutes. The supernatant was removed and the cell pellet was resuspended in an appropriate amount of 1xPBS to obtain a final concentration of $2x10^6$ cells per milliliter. To the wells containing the compound, 50ul of the cells in 1xPBS ($1x10^5$ cells) were added. The plate was incubated on a shaker for 15 minutes at room temperature. The detection buffer containing the tracer cAMP was prepared. In 11ml of detection buffer (NEN: #SMP004A), 50ul (equal to 1uCi) of [^{125}I]cAMP (NEN: #SMP004A) was added. Following incubation, 50ul of this detection buffer containing tracer cAMP was added to each well. The plate was placed on a shaker and incubated at room temperature for two hours. Finally, the solution from the wells of the plate were aspirated and the flashplate was counted using the Wallac MicroBeta plate reader.

Appendix B2

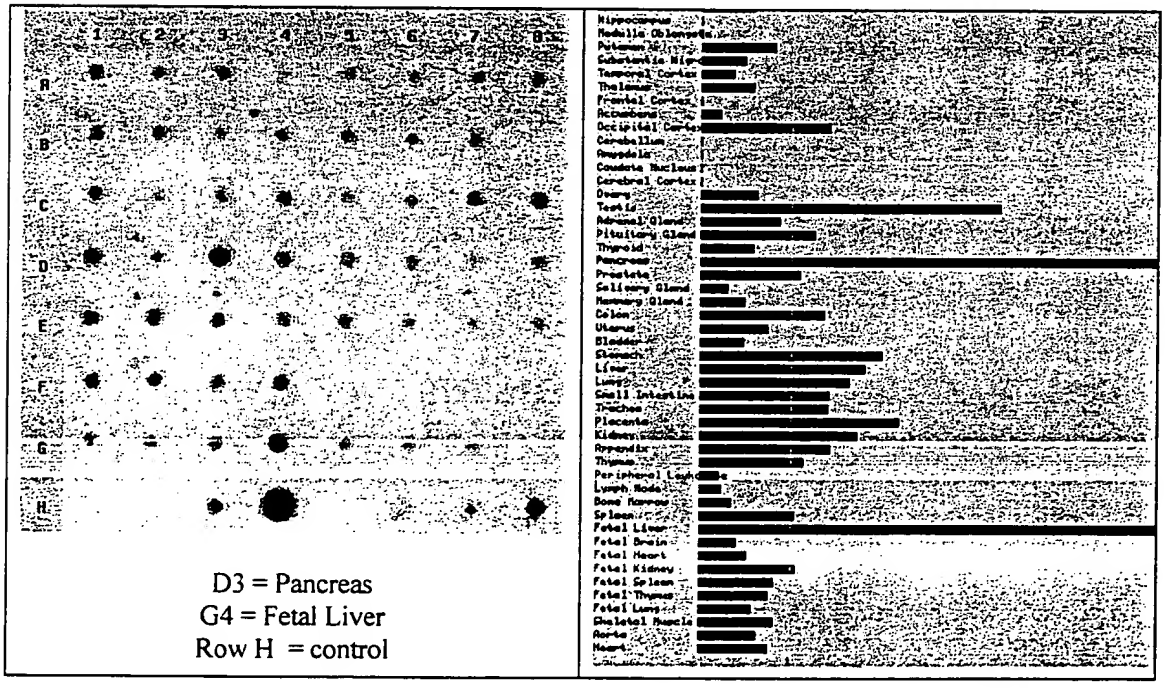


Appendix B3

Dot-Blot Protocol

19AJ PCR fragment was used as a probe: radiolabeled probe generated using this and a Prime-IT™ Random Primer Labeling Kit (Stratagene) was utilized, according to manufacturer's instructions. A human RNA Master Blot™ (Clontech) was hybridized with the human 19AJ radiolabeled probe and washed under stringent conditions according to manufacuter's instructions. The blot was exposed to Kodak BioMax Autoradiography film overnight at -80°C.

Appendix B4



Appendix B5

RT-PCR Protocol

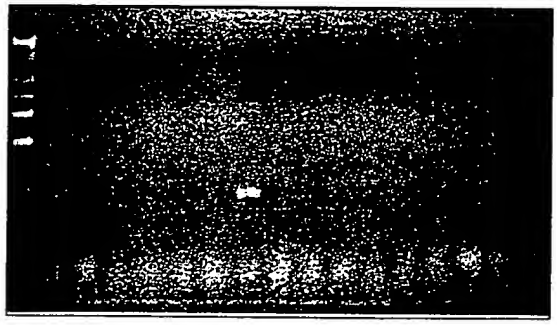
To ascertain the tissue distribution of 19AJ mRNA, RT-PCR was performed using 19AJ-specific primers and human multiple tissue cDNA panels (MTC, Clontech) as templates. Taq DNA polymerase (Stratagene) was utilized for the PCR reaction, using the following reaction cycles in a 40 µl reaction: 94°C, 2 minutes; 94°C, 15 seconds, 55°C, 30 seconds;72°C, 1 minute; 72°C, 10 minutes. Primers were as follows:

5'-GACAGGTACCTTGCCATCAAG-3' and

5'-CTGCACAATGCCAGTGATAAGG-3'.

20µl of the reaction were loaded onto a 1% agarose gel.

Appendix B6



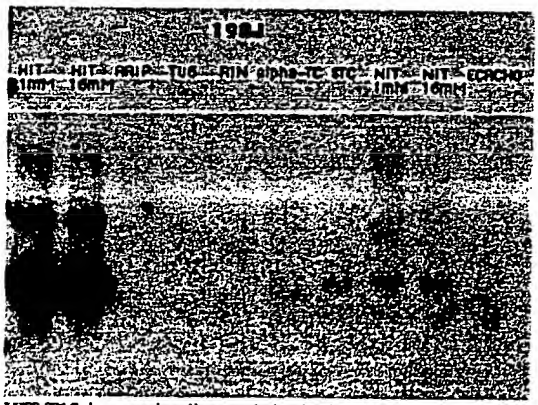
1 A B C D E FG H I J K L M N O P

G = Pancreas

Appendix B7

Total RNA from several exocrine and endocrine pancreatic cell lines were isolated using TRIzol reagent (Gibco/BRL, Cat #15596-018) according to manufacturer's instructions. After electrophoreseis in a 1% agarose/formaldehyde gel, the RNA was transferred to a nylon membrane using standard protocols. A ^{32}P-labelled 19AJ probe was synthesized using a DNA fragment corresponding precisely to the entire coding sequence and a Prime It II Random Primer Labeling Kit (Stratagene, Cat. #300385) according to manufacturer's instructions. Hybridization was performed using ExpressHyb Solution (Clontech, Cat.#8015-2) supplemented with 100ug/ml salmon sperm DNA as follows. The membrane containing the separated RNA samples were first incubated with ExpressHyb solution at 65°C for 1 hour. The ^{32}P-labeled 19AJ DNA probe was denatured by boiling for 2 min, placed on ice for 5 min and then transferred into the ExpressHyb solution bathing the membrane. After an overnight incubation at 65°C, the membrane was removed from the hybridization and washed four times for 15 min each in 2XSSC/1% SDS at 65°C, followed by two washes for 15 min each in 0.1XSSC/0.5% SDS at 55°C. Excess moisture was removed from the blot by gentle shaking, after which the blot was wrapped in plastic and exposed to film overnight at -80°C. RNA blots evidenced that 19AJ was abundantly expressed in glucose responsive β-cell lines HIT-15 and NIT-1.

Appendix B8



HIT-T15: hamster insulinoma-derived, glucose-responsive
NIT-1: mouse insulinoma-derived, glucose-responsive
Tu6: rat insulinoma-derived, also produces somatostatin
RIN-5AH: rat insulinoma-derived
αTC-1: mouse glucagonoma derived (also has a small fraction of insulin-producing cells)
STC-1: mouse intestinal endocrine cell line, makes glucagon, GLP-1
ARIP: rat pancreatic exocrine ductal line

Appendix B9

Insulin Assay Protocol

To measure insulin secretion from rodent insulin-producing cell lines, cells were first cultured overnight in serum-free, glucose-deficient media. The following morning, the cells were then placed in the same media supplemented with either 1 mM or 16 mM glucose. In some cases, the cells were also incubated with the 19AJ agonist Cmd A at a concentration of 10 μM. After an incubation of 5 hours, the media was collected and analyzed for insulin content using a Rat Insulin Enzymeimmunoassay (EIA) System (Amersham Pharmacia Biotech, Cat. No. RPN 2567). Typically, the assay was performed using multiple dilutions of sample media in order to ensure that the sample measurements fell within the boundaries of the standard curve (generated using known amounts of insulin), as recommended by the manufacturer.

Appendix B10